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TITLE: Preclinical Evaluation of Serine/Threonine Kinase Inhibitors Against Prostate Cancer Metastases

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Bone is a rich store of growth factors that stimulate metastatic cancer cells. TGFbeta in bone increases tumor secretion of factors that activate bone remodeling, fueling a vicious cycle, driving growth and survival of prostate bone metastases. TGFbeta signals through a receptor with two serine/threonine kinases subunits and, further downstream, partly through another serine/threonine kinase, p38 MAPK. We hypothesized that kinase inhibitors would reduce prostate cancer metastasis to bone. Two orally active inhibitors were tested in animal models of prostate cancer bone metastases. Aim 1 testeda TGFbeta receptor I kinase inhibitor against two human prostate cancer models of skeletal metastases in mice. Aim 2 found a molecular target of the inhibitors in prostate cancer cells: PMEPA1. Aim 3 was totest the efficacy of combined TGFbeta receptor I and p38 MAPK inhibitors against two prostate cancer models in vivo, but only the former class of drug was effective. TGFbeta inhibitors may be clinically useful against osteolytic bone metastases.

15. SUBJECT TERMS
Bone metastases, prostate cancer, TGF-beta, PMEPA1

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GENERAL INTRODUCTION

Prostate cancer has a propensity to grow in the skeleton and cause significant morbidity. Once housed in bone, prostate cancer is incurable. Bone is a rich storehouse of growth factors, which stimulate signaling in metastatic cancer cells. Bone-derived TGFβ increases tumor secretion of factors that activate bone remodeling, fueling a vicious cycle, which drives the growth and survival of prostate bone metastases. In prostate cancer cells, TGFβ signals through two receptor subunits and, further downstream, p38 MAP kinase. Hypothesis: *TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase pathway. TGFβ and/or p38MAP kinase signaling inhibitors will reduce the development and progression of prostate cancer bone metastases to bone.* Two orally active inhibitors of these serine/threonine kinases will be tested in an animal model of prostate cancer bone metastases. We propose three Specific Aims. **Aim 1**: To test a TGFβRI kinase inhibitor and a p38 MAPK inhibitor against three human prostate cancer models of skeletal metastasis in mice. **Aim 2**: To determine the molecular targets of these inhibitors in prostate cancer cells *in vitro* and test their impact on tumor growth and bone metastases *in vivo*. **Aim 3**: To test the efficacy of combined TGFβRI and p38 MAP kinase inhibitors against three prostate cancer models *in vivo*.

Summary of basic progress after three years:

- TGFβRI kinase inhibitor *effective* against osteolytic bone metastases
- TGFβRI kinase inhibitor *ineffective* and possibly deleterious against osteoblastic bone metastases.
- p38 MAPK inhibitor *ineffective* in several models of bone metastases. Additional experiments with this class of inhibitor and the combined treatments proposed in Aim 3 have consequently been abandoned.
- Inhibition of TGFβ signaling without effect on growth of tumors at soft tissue sites
- PMEPA1 identified as major target gene of TGFβ and role of PMEPA1 as regulator of TGFβ signaling found.

To be Completed in year four under No Cost Extension:

- Completion of histological analyses of animal models with bone metastases +/- treatments
- Test of PMEPA1 function by overexpression in prostate cancer cells and introduction into mouse model of bone metastasis

Tasks Completed: Included as an Appendic at the end of this report is a reproduction of the original Statement of Work, with the addition of a status summary for each of the 23 originally-proposed Tasks

BODY OF REPORT

Original Background. The skeleton is a major site of metastasis by advanced prostate cancer. In a recent year 220,900 cases of prostate cancer were diagnosed in the United States, where it is now the most commonly diagnosed cancer and the second most common cause of cancer mortality in men, with 28,900 deaths (Crawford, 2003). One fourth of diagnosed patients will die from

the disease, the majority of them with metastases to the skeleton. Once cancer becomes housed in bone, it is incurable. The average survival from time of diagnosis of skeletal metastases in prostate cancer patients is 40 months. When prostate tumor cells metastasize to the skeleton, the most common response is osteoblastic: characterized by net formation of disorganized new bone, which results in fractures, severe and intractable bone pain, and nerve compression. Metastasis to bone thus causes prolonged, serious morbidity for many prostate cancer patients. Treatment to prevent or halt the progression of bone metastases (Reddi et al, 2003; O'Keefe and Guise, 2003). would increase survival and improve quality of life for men with prostate cancer

Transforming growth factor-β in cancer is a two-edged sword. TGFβ is a growth inhibitor and a tumor suppressor at early stages of the oncogenic cascade. However, advanced cancers often lose the growth inhibition by TGFβ but continue to respond to the factor. The net effect is that TGFβ is a metastasis enhancer for advanced cancers. Since bone is a major source of active TGFβ, the factor plays a crucial role in the vicious cycle of bone metastases. Blockade of the TGFβ pathway effectively decreases metastases in several animal models (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002).

Transforming growth factor-β in bone is released from mineralized matrix in active form by osteoclastic resorption (Dallas et al, 2002), which is very prominent in prostate cancer metastases. TGFβ acts on tumor cells to increase the secretion of factors that inappropriately stimulate bone cells (Chirgwin & Guise, 2003a,b). The interactions between bone and cancer constitute a vicious cycle, which enhances skeletal metastases (Mundy, 2002). Extensive data show that TGFβ is a major bone-derived factor responsible for driving the vicious cycle of cancer metastases in bone. TGFβ increases tumor secretion of factors such as endothelin-1, IL-6, IL-11, PTHrP, and VEGF. These factors stimulate both osteoblastic synthesis of disorganized new bone and osteolytic destruction of the skeleton adjacent to tumor cells. The cellular and molecular components of the vicious cycle between tumor and bone offer opportunities for therapeutic intervention to decrease skeletal metastases (Coleman, 2002; Guise & Chirgwin, 2003a). TGFβ in particular is an important target for intervention against prostate cancer skeletal metastases.

Therapy to block TGFβ signaling in bone metastases. Previous work has demonstrated the effectiveness of TGF-beta inhibition to decrease metastases, but these experiments have used protein-based treatment or ex vivo manipulations of the tumor cells (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002). Orally active small-molecule inhibitors of the TGFβ pathway would be much more practical. This proposal will test two inhibitors of serine/threonine kinases. The first directly targets the TGFβ receptor kinase. The second targets p38 MAP kinase, which is a major downstream effector of TGFβ signaling in cancer cells. Both targets are serine/threonine kinases. Our preliminary data show that inhibition of TGFβ signaling is effective in an animal model of cancer

bone metastases. The work proposed will test the two serine/theronoine kinases inhibitors in animal models of human prostate cancer in bone: one in which the response is osteolytic, two others in which it is osteoblastic. The experiments proposed will rapidly provide the preclinical data necessary for these two drugs to be placed in clinical trails for prostate cancer bone metastases.

Hypotheses: 1) TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase. Specific serine/threonine kinase small-molecule inhibitors of the type I TGFβ receptor kinase and of p38 MAP kinase will reduce the development and progression of prostate cancer metastases to bone, due to either osteoblastic or osteolytic diseases. 2) Orally active inhibitors of these serine/threonine kinases will be effective in animal models of prostate cancer bone metastases to decrease metastases and tumor burden and to increase survival. 3) The two drugs may be more effective in combination than singly, if p38 MAP kinase also mediates TGFβ-independent metastatic functions. 4) Specific targets of TGFβ signaling in prostate cancer cells contribute directly to the bone phenotype of metastases. One such factor may be the type I membrane protein PMEPA1, which is regulated by TGFβ and expressed by prostate cancers. 5) Expression of PMEPA1 on the surface of cancer cells will increase the development and progression of prostate cancer metastases to bone.

Specific Aim 1: To determine the effect of TGFβRI kinase or p38 MAPK blockade separately against 3 human prostate cancer models of skeletal metastasis in mice (hypotheses 1 & 2). Data provided below were also included in the previous (second) annual progress report,

Summary of Results and Progress 10/06-10/07: We tested the TGFβRI kinase, SD-208, on the development and progression of bone metastases due to PC-3 and LuCAP23.1 prostate cancers. This aim has taken longer than originally planned because we had to determine long-term pharmacokinetics for drug delivery in the food. 50-100 mg/kg of SD-208 added to food result in drug levels effective in a mouse model of breast cancer metastases to bone. In the prostate cancer models SD-208 reduced osteolytic bone metastases due to PC-3, but increased osteoblastic bone metastases due to LuCAP23.1. There was no effect on the mixed tumor, C42B, which is unresponsive to TGFβ. The p38MAP kinase inhibitor, SD-282 increased bone metastases due to PC-3 prostate cancer and had no effect on LuCAP23.1 or C42B. Since SD-282 had no positive effects in 3 models, we will not pursue Aim 3, which was to combine SD-208 and SD-282 treatments. Since SD-282 lacked efficacy, no further experiments were carried out to test its effects on soft-tissue growth, and SD-208 remains to be tested only against subcutaneous growth of PC3 and LuCaP23.1 xenografts.

Specific Aim 2: To determine the molecular targets of the inhibitors in prostate cancer cells in vitro by gene array analysis (hypothesis 4). The role of an already-identified target of TGFβ, PMEPA1, will be tested in the animal models by overexpressing it in 2 prostate cancer cell lines (hypothesis 5).

Summary of Results and Progress 10/06-10/07: Gene array targets of TGFβ on PC-3 prostate cancer were validated by quantitative real-time PCR and

were described in the progress report for year one. We found that PMEPA1 is expressed in three different isoforms, which may have different subcellular localizations and biological activities,Stabel knockcdown and overexpressing cell lines have been made. They will be tested in year 04 in the bone metastasis model. Characterization of the complex PMEPA1 promoter is complete.

Specific Aim 3: To test the efficacy of combined TβRI and p38 MAPK inhibitors against 3 prostate cancer models in vivo (hypothesis 3).

Results and Progress: Since the p38 MAPK inhibitor was entirely without benefit in Aim 1, combination trials with this drug would be a pointless waste of research animals and this Aim will not be pursued.

RESULTS:

Aim 1*: A p38 MAP kinase inhibitor **increased** osteolytic bone metastases due to PC-3 prostate cancer.* The p38MAP kinase inhibitor, SD-282, accelerated development of osteolytic lesions due to PC-3 prostate cancer (**Figure 1**). Mice were treated with SD-282 when bone metastases were identified on radiographs, at 4 weeks. Doses used were based on pharmacokinetic studies done by the manufacturer, Scios, Inc. We found similar results using the osteolytic breast cancer model, MDA-MB-231: p38MAP kinase inhibition increased osteolytic bone metastases (not shown). With LuCAP23.1 and C42B cells, there was no effect of SD-282 on either group. Quantitative bone histomorphometry is underway for these experiments.

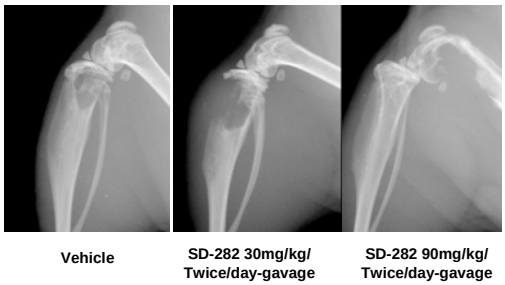

Figure 1: **p38MAPK inhibitor SD-282** and PC-3 bone metastases. Representative radio-graphs (4X magnification) of legs from mice with PC-3 prostate cancer cells 8wks post tumor inoculation. Larger osteolytic bone lesions in mice treated with SD-282 (right 2 panels) compared to vehicle (left panel).

TGFβRI kinase inhibitor reduced osteolytic bone metastases due to PC-3 prostate cancer. In contrast (and similar to results observed with osteolytic breast cancer model, MDA-MB-231 but not shown), the TGFβRI

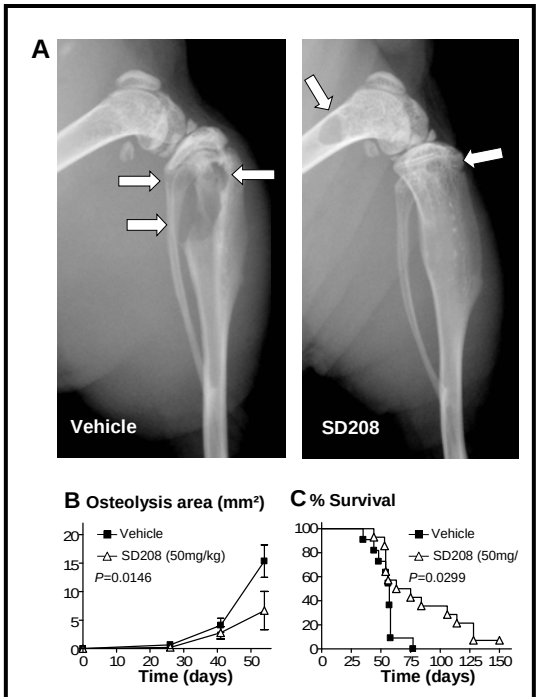

Figure 2. SD-208 prevents PC-3 bone metastases. Mice inoculated i.c. with 10^5 cells PC-3 cells & given 50mg/kg SD-208 (n=14) or vehicle (n=11) 2d prior to inoculation. **A**. X-rays (4X mag) of distal femur & proximal tibia. Arrows point to osteolytic lesions. **B**. Osteolytic lesion area. Ave ± SE, by 2-way ANOVA. **C.** Kaplan-Meier survival curves.

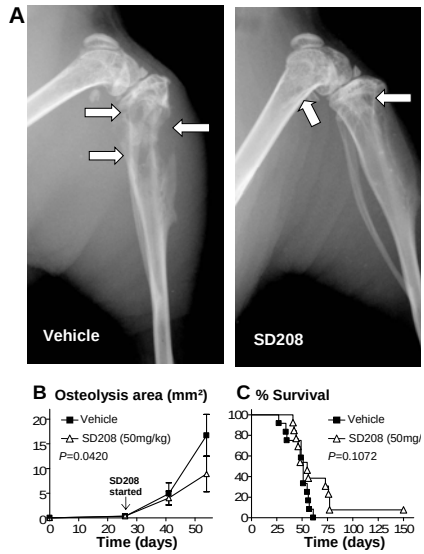

Figure 3: Treatment of established PC-3 bone metastases with SD-208. As in **Figure 2**, except mice given 50mg/kg SD-208 (n=13) or vehicle (n=12) beginning at d28, when metastases seen on X-ray.

kinase inhibitor, SD-208, reduced osteolytic bone metastases and improved survival in mice bearing PC-3 prostate cancers (both treatment and prevention protocols (**Figure 2, 3**).

Conclusion: Taken together with data that a p38MAPK inhibitor was ineffective and even increased PC-3 and MDA-MB-231 bone metastases, it may be better to target the Smad pathway than total TGFβ signaling. The latter may be less effective, if downstream p38MAP kinase blockade adversely affects bone metastases.

Part 2: TGFβRI kinase inhibitor accelerates osteoblastic bone metastases due to prostate cancer LuCAP23.1. Since our prior studies included only osteolytic bone metastases models, and TGFβ has been implicated in the pathogenesis of prostate cancer metastases to bone, we tested SD-208 in a model of human prostate cancer, LuCAP23.1, which grows as osteoblastic lesions when directly injected into bone. LuCAP23.1 (obtained from our collaborator, Robert Vessella, University of Washington) is an androgen-sensitive, PSA-producing human tumor derived from an osteoblastic bone metastasis. It causes osteoblastic lesions in 10-12 weeks. **Figure 4** shows representative radiographs from LuCAP23.1 bearing mice, treated with SD-208 at 20 or 60 mg/kg/day after inoculation into bone. Drug was started at 12 weeks, after lesions were evident by radiographs. The experiment shows clearly that *osteoblastic bone metastases can be accelerated* by treatment with SD-208. Thus TGFβRI kinase inhibition may worsen osteoblastic metastases. We have other data that SD-208 has direct effects to increase osteoblast activity. This 'host' response to the drug could *accelerate* osteoblastic disease in prostate cancer. LuCAP23.1 is a xenograft and cannot be studied in culture as a cell line. In year 04 we will test TGFβ inhibition on another osteoblastic models of breast and prostate cancer. ZR-75-1 is a human breast cancer that causes osteoblastic metastases due to tumor production of ET-1 (Yin et al 2003). We tested SD-208 on bone metastases due to the mixed osteolytic/osteoblastic tumor C42B. No effect was observed in treated compared to control animals. Quantitative histomorphometry is underway on bones from all experiments.

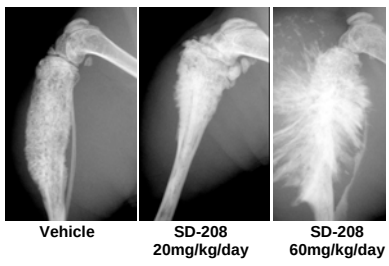

Figure 4: SD-208 increases osteoblastic bone metastases: Representative radiographs (4X mag) of legs of mice with intratibial LuCap23.1 prostate cancer xenograft after 18wks. Larger lesions in mice treated with SD-208 (right) vs vehicle.

Aim 2: Identification of PMEPA1 as a major target gene of TGFbeta in metastatic cancer cells and analysis of role of PMEPA1 in TGFbeta signaling.

We previously identified the PMEPA1 gene as the most highly upregulated gene in prostate cancer cells treated with TGFbeta. The background on this protein is provided in the previous report. The protein sequence suggests that the protein could regulate intracellular signaling in particular via the TGFbeta pathway. Data are now provided to uspport this hypothesis.

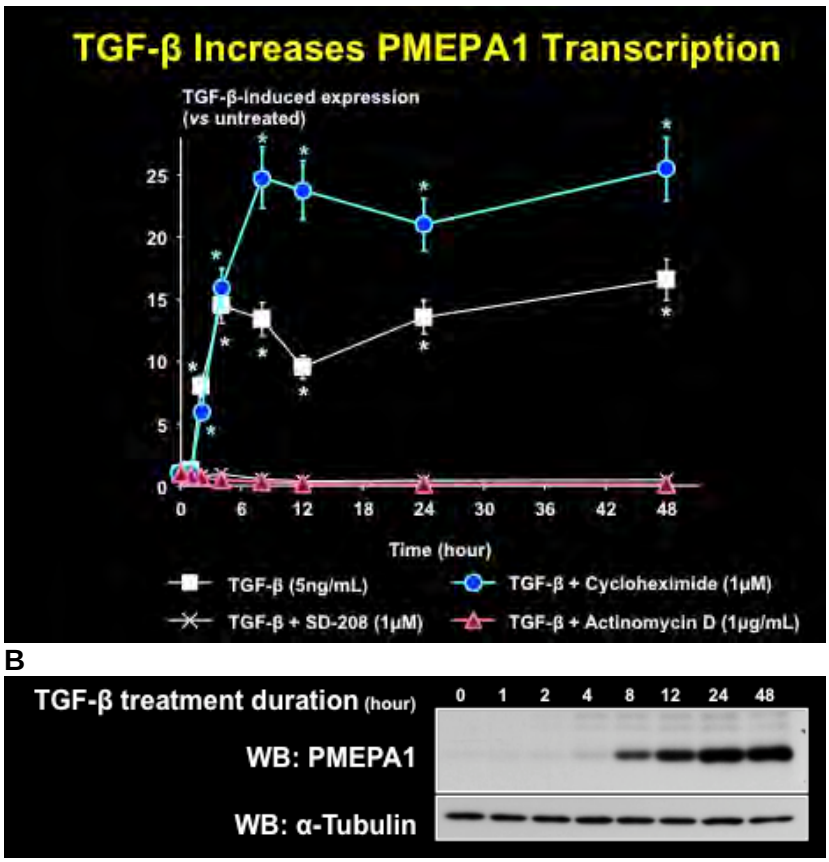

Figure 5: **PMEPA1 is expressed in cell lines that cause bone metastases**. RT-PCR, showed that PMEPA1 was expressed in different PrCa cell lines, LnCaP, C4-2B and DU145, BrCa MDA-MB-231 and lung adenocarcinoma A549. The hepatocarcinoma HepG2 did not express detectable PMEPA1. When cells were treated with TGF-β for 24 hours, PMEPA1 mRNA was increased in most of the cells but not in LnCaP or in its derivative C4-2B. However LnCaP and C4-2B are TGF-β insensitive.

PMEPA1 is expressed in cell lines that cause bone metastases. Using RT-PCR, we found that PMEPA1 is expressed in different PrCa cell lines, LnCa, C4-2B and DU145, the BrCa cells MDA-MB-231 and the lung adenocarcinoma A549. The hepatocarcinoma HepG2 did not express detectable PMEPA1. When cells were treated with TGF-β for 24 hours, PMEPA1 mRNA was increased in most of the cells but not in LnCaP or in its subclone C4-2B (**Figure 5**). However we tested (as others have previously reported in the literature) that the LnCaP and C4-2B cell lines are TGF-β insensitive.

TGF-β increases PMEPA1 transcription and protein. We validated the increase of PMEPA1 expression induced by TGF-β in PC-3 cells. PMEPA1 mRNA was quickly increased by TGF-β and reached a peak by 4 hours. This TGF-β induction was prevented by adding the specific TGF-β receptor inhibitor, SD-208. We used classical cycloheximide and actinomycin-D inhibitor treatments to determine if the effects were transcriptional or translational. We found that the translation inhibitor cycloheximide did not block TGF-β induction of PMEPA1, while the transcription inhibitor actinomycin D prevented the increase of PMEPA1 mRNA. The results (**Figure 6**) suggest that TGF-β regulates PMEPA1 expression through transcriptional control. Western blot (bottom panel) showed PMEPA1 protein increase at 48 hours.

The PMEPA1 gene covers 63kb. Alternative splicing and multiple transcription starts give rise to 4 different mRNA variants. These mRNA encodes 3 different protein

isoforms. Isoforms a & b contain a transmembrane domain, while isoform c, the shortest, is cytosolic (**Figure 7**).

TGF-β induces the cytosolic (c) isoform of PMEPA1 in PC-3 cells: The isoforms of PMEPA1 were cloned and expressed in COS cells (left panel). Western blot showed that the PMEPA1 antibody detected all isoforms (**Figure 8**). In PC-3 cells, only the cytosolic isoform was induced by TGF-β (right panel)

Selection of shRNA vectors which knock-down all isoforms of PMEPA1. We validated a vector expressing a short hairpin RNA against the 3' extremity of PMEPA1 mRNA, analog to all variants. Using real-time PCR, we showed that in CHO cells transfected to express one of the PMEPA1 isoform, there was a 90% decrease of all corresponding PMEPA1 mRNA (**Figure 9**). An empty vector or a vector expressing a non-specific shRNA had no effect on PMEPA1 mRNA quantity. Similarly, using Western Blot, the shRNA against PMEPA1 specifically decreased PMEPA1 protein quantity regardless of the isoform.

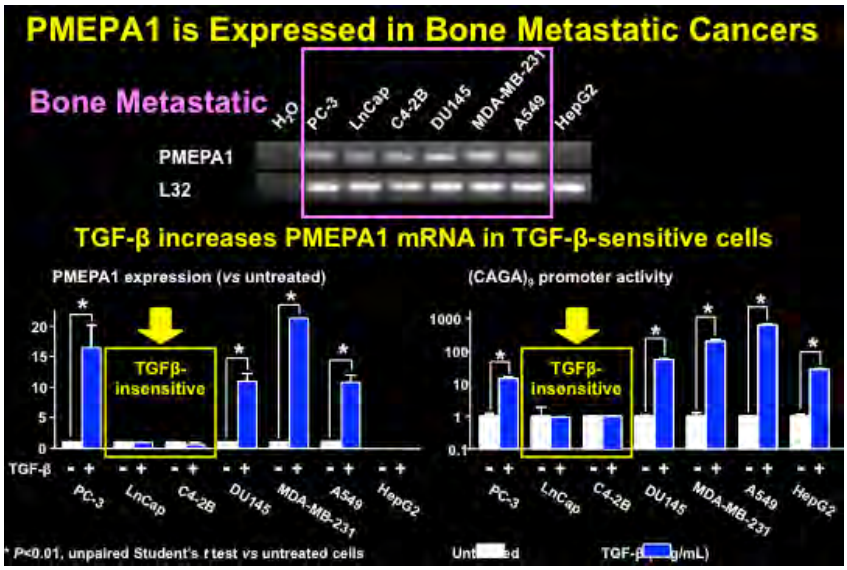

Figure 6: TGF-β increases PMEPA1 transcription and protein. PMEPA1 expression was induced by TGF-β in PC-3 cells. PMEPA1 mRNA quickly increased by TGF-β and reached a peak by 4 hours. TGF-β induction was prevented by adding the specific TGF-β receptor inhibitor, SD-208. Classical cycloheximide and actinomycin-D treatments used to determine if the effects were transcriptional or translational. The translation inhibitor cycloheximide did not block TGF-β induction of PMEPA1, while the transcription inhibitor actinomycin D prevented the increase of PMEPA1 mRNA. The results suggest that TGF-β regulates PMEPA1 expression via transcription. Western blot (bottom panel) showed PMEPA1 protein increase at 48hrs.

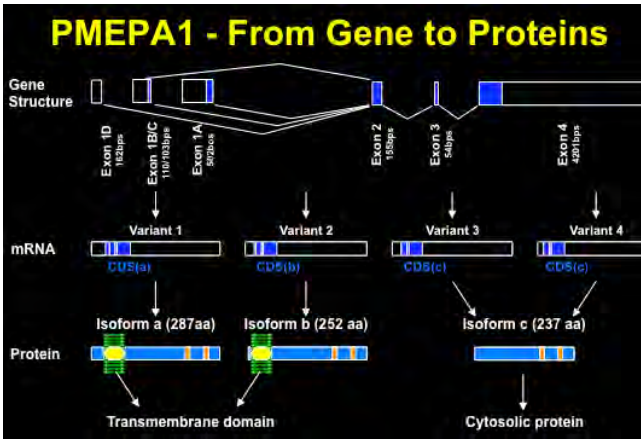

Figure 7: The PMEPA1 gene covers 63kb. Alternative splicing and multiple transcription starts give rise to 4 different mRNA variants. These mRNA encodes 3 different protein isoforms. Isoforms a & b contain a transmembrane domain, while isoform c, the shortest, is cytosolic.

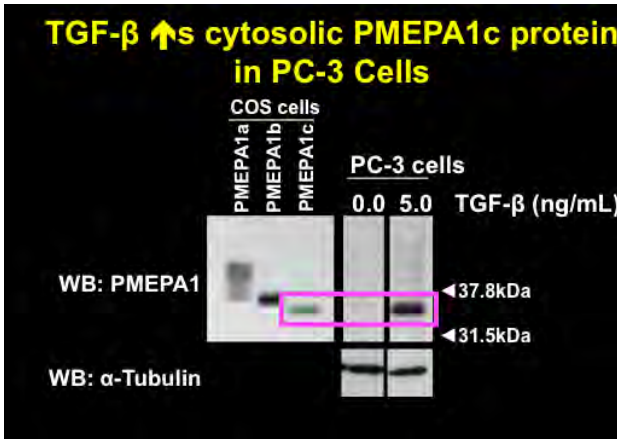

Figure 8: TGF-β induces the cytosolic form of PMEPA1 in PC-3 cells: The isoforms of PMEPA1 were cloned and expressed in COS cells (left panel). Western blot showed that the PMEPA1 antibody detected all isoforms. In PC-3 cells, only the cytosolic isoform was induced by TGF-β (right panel).

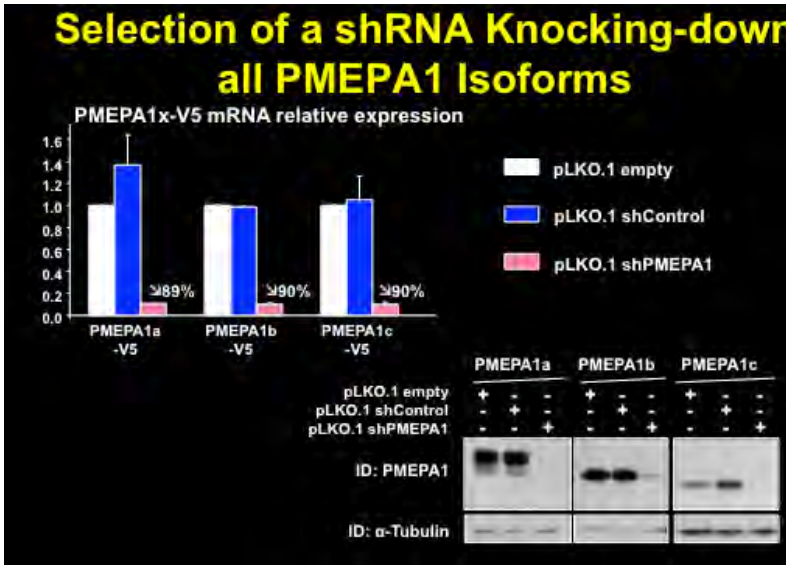

Figure 9: Selection of shRNA vectors for knock-down all isoforms of PMEPA1. Validation of vector expressing a short hairpin RNA against the 3' end of PMEPA1 mRNA, common to all variants. By PCR (upper panel) of CHO cells transfected to express one of the PMEPA1 isoform, there was a 90% decrease of all corresponding PMEPA1 mRNAs. Empty vector or one expressing a non-specific shRNA had no effect on PMEPA1 mRNA quantity. By western blot (lower panel) the shRNA against PMEPA1 specifically decreased PMEPA1 protein quantity regardless of the isoform.

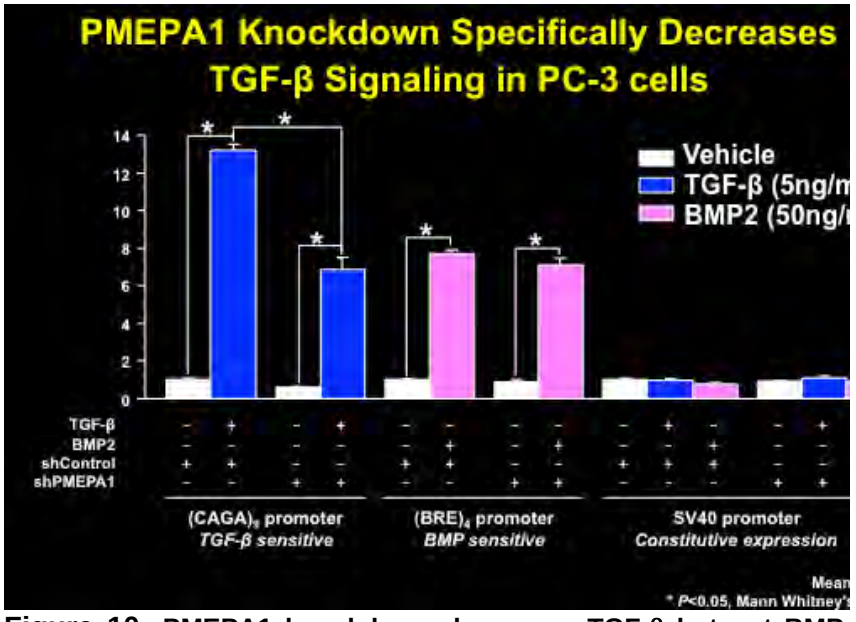

Figure 10: PMEPA1 knockdown decreases TGF-β but not BMP signaling. TGF-β signaling in PC-3 tested with (CAGA)9 promoter when cells were transfected with a vector expressing either a non-targeting shRNA or an shRNA against PMEPA1. Knockdown of PMEPA1 in PC-3 cells significantly decreased (CAGA)9 promoter activity induced by TGF-β. This result suggests that PMEPA1 in PC-3 cells increases TGF-β signaling. There was no effect on BMP promoter activity as assessed by BRE activity or an unrelated SV40 promoter.

PMEPA1 knockdown decreases TGF-β but not BMP signaling. We tested TGF-β signaling in PC-3 using the (CAGA)9 promoter when the cells were transfected with a vector expressing either a non-targeting shRNA or an shRNA against PMEPA1. Knockdown of PMEPA1 in PC-3 cells, induced a significant decrease of the (CAGA)9 promoter activity induced by TGF-β. This result suggests that PMEPA1 in PC-3 cells increases TGF-β signaling (**Figure 10**). There was no effect on BMP promoter activity as assessed by BRE activity or an unrelated SV40 promoter.

Model for PMEPA1 role in bone metastases: We hypothesize that PMEPA1, when induced by TGF-β at the site of bone metastases, interacts with Smurf proteins, to prevent the degradation of Smads and the TβR. This results in a sustained TGF-β signaling and an increase of bone metastases development (**Figure 11**). PMEPA1 could also directly affect Smad activity when interacting with them by a mechanism that remains to be elucidated. Experiments are underway to test the function of PMEPA1 protein expression on TGFbeta signaling in prostate cancer bone metastases.

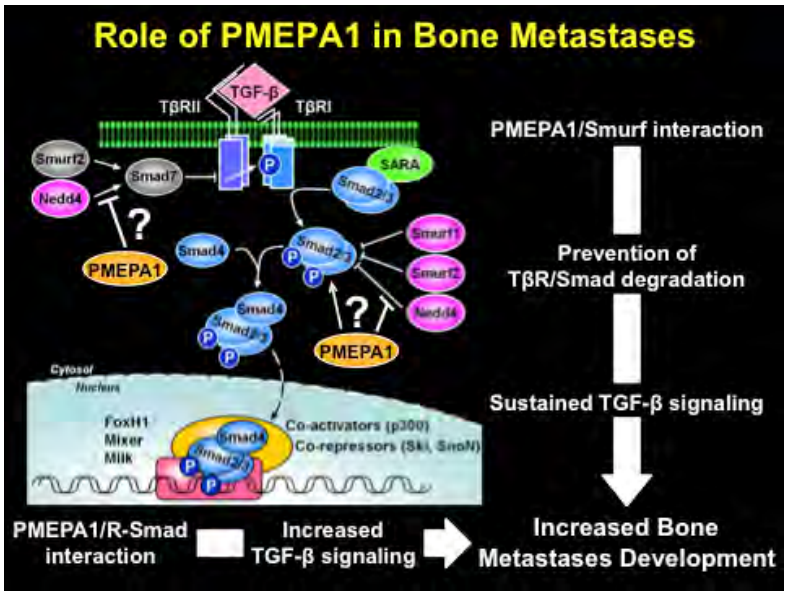

Figure 11: Model for PMEPA1 role in bone metastases: PMEPA1, when induced by TGF-β at the site of bone metastases, may interact with Smurf proteins to prevent the degradation of Smads and the TβRs. This results in sustained TGF-β signaling and increased bone metastases. PMEPA1 could also directly affect Smad activity when interacting with them by a mechanism that remains to be elucidated.

KEY RESEARCH ACCOMPLISHMENTS October 2006 – October 2007

Aim 1:

- TGFbeta RI kinase inhibitor, SD-208, effective against bone metastases due to PC3 prostate cancer model
- SD-208 ineffective against C42B prostate cancer bone metastasis model
- SD-208 ineffective, possibly deleterious against LuCaP23.1 prostate cancer bone metastasis xenograft model
- The p38 MAP kinase inhibitor SD-282 ineffective against all three prostate cancer bone metastasis models

Aim 2:

- TGFbeta regulation of PMEPA1 promoter determined at molecular level
- Role of three protein isoforms of PMEPA1 in TGFbeta signaling potentiation shown

REPORTABLE OUTCOMES

Presentations: October 2006 – October 2007

1. Molecular mechanisms of bone metastases: Osteolytic and osteoblastic. Italian Cancer Society Meeting, Bari, Italy, Oct 2006.
2. TGFβ in bone metastases: Pathophysiology to treatment. Visiting Professor, University of Minnesota, Minneapolis, MN, Dec 2006.
3. TGFβ signaling in breast cancer bone metastases: Friend or foe? Cancer and Bone Society Meeting, San Antonio, TX, Dec 2006.
4. Molecular mechanisms of bone metastases: Insight into therapy. Endocrine Grand Rounds, University of Texas Health Science Center at San Antonio, TX, Dec 2006.
5. Skeletal complications of cancer and cancer treatment. Bone Club, San Antonio, TX, Dec 2006.
6. Skeletal health in the cancer patient. Maine State Osteoporosis Meeting, Sugarloaf, ME, Jan 2007.
7. Effects of bisphosphonates on tumor cells. Consensus on Bone Loss in Cancer Patients on Aromatase Inhibitors, Geneva, Switzerland, Feb 2007.
8. TGFβ in bone metastases: Pathophysiology to treatment. Institute for Molecular Medicine, University of Lisbon, Lisbon, Portugal, Mar 2007.
9. RANK ligand in prostate cancer metastases to bone. Medical Grand Rounds, Hospital Santa Ana, University of Lisbon, Lisbon, Portugal, Mar 2007.
10. Skeletal health in the cancer patient. Endocrine Grand Rounds, Oregon Health Sciences University, Portland, OR, Apr 2007.
11. TGFβ signaling in breast cancer bone metastases: Friend or foe? Research Seminar, Oregon Health Sciences University, Portland, OR, Apr 2007.
12. TGFβ in bone metastases: Pathophysiology to treatment. Endocrinology Grand Rounds, Mount Sinai School of Medicine, New York, NY, Apr 2007.
13. TGFβ signaling in breast cancer bone metastases: Friend or foe? Advances in Mineral Metabolism Meeting, Snowmass, CO, Apr 2007.
14. TGFβ signaling in cancer metastases to bone: Friend or foe? Cleveland Clinic, Cleveland, OH, Apr 2007.
15. Biology of bone metastases. FASEB Meeting, Washington, DC, Apr 2007.
16. Endothelins in pathologic and normal bone remodeling. Bone Club, University of Pittsburgh, Pittsburgh, PA, May 2007.
17. Endothelins in pathologic and normal bone remodeling. Research Seminar, Wyeth, Collegeville, PA, May 2007.
18. TGFβ in cancer and bone: Friend or foe? Research Seminar, University of Rochester, Rochester, NY, Jul 2007.
19. Endothelins: Cancer, bone and beyond. Research Seminar, Vanderbilt University, Nashville, TN, Aug 2007.
20. TGFβ-regulated genes in prostate cancer. Department of Defense IMPACT meeting, Atlanta, GA, Sep 2007.
21. Mechanisms and treatment of bone metastases. International Carcinoid Meeting, Norfolk, VA, Sep 2007.
22. TGFβ signaling in cancer and bone: Friend or foe? University of Alabama, Birmingham, AL, Oct 2007.
23. Cancer and bone. National Academy of Continuing Medical Education Meeting, Fort Lauderdale, FL, Sep 2007.
24. TGFβ signaling in bone metastases due to breast cancer, prostate cancer and

melanoma. Skeletal Complications of Malignancy Meeting, Philadelphia, PA, Oct 2007.

25. TGFβ in cancer and bone: Friend or foe? University of Miami, Miami, FL, Oct 2007.

Publications: October 2006 – October 2007

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14. Bartholin L, **Guise TA**. TGFβ in breast cancer osteolysis. IN: Textbook: TGFβ in Health and Disease; S Jakalew, Ed; Ch 7, 2007.

Funding: October 2006 – October 2007

Active Grant Awards

1. National Institutes of Health (NCI), "TGFβ in the bone microenvironment: role in metastases" (R01-CA69158-12; Guise, PI, 20% effort). Awarded: 12/01/07-11/30/12.
2. National Institutes of Health (NIDDK), "Endothelin-1 in normal and pathological bone remodeling" (R01DK067333; Guise, PI; 20% effort).
3. National Institutes of Health (NIDDK), "Prostate cancer metastasis to bone: Role of adrenomedullin" (R01 DK065837) (Guise, PI; 20% effort). Awarded: 04/01/05-03/30/10.
4. Prostate Cancer Foundation, "Inhibition of prostate cancer bone metastases with endothelin receptor blockade plus bisphosphonate antiresorptive: preclinical testing and molecular mechanisms." Awarded: 02/01/04-01/31/08.
5. US Army Prostate Cancer Program Idea Award PC040341, "Preclinical evaluation of serine/threonine kinase inhibitors against prostate cancer metastases" (Guise, PI, 15% effort). Awarded: 10/01/04-09/30/08. **THIS AWARD**.
6. V-Foundation, "Effects of a high bone turnover state induced by estrogen deficiency on the development and progression of breast cancer and bone metastases." Awarded: 10/01/04-09/30/08.
7. Mary K. Ash Foundation, "Inhibition of breast cancer bone metastases with anti-hypoxic treatment" (Guise, PI). Awarded: 07/01/05-06/30/08.
8. P01 (NIH, NCI), "Signaling and Progression in Prostate Cancer: Core D: Tissue Analysis Laboratory" (Theodorescu, PI; Guise Project Co-Leader, Core C 10% effort). Awarded: 06/01/04-05/30/09.
9. P01 (NIH, NCI), "Signaling and Progression in Prostate Cancer: Core B: Cell culture, animal models and imaging" (Theodorescu, PI; Guise Project Leader, Core B 10% effort). Awarded: 06/01/04-05/30/09.

Recent Previous Grant Awards

1. Department of Defense, subcontract from Emory University: "Targeting the lethal phenotype of metastatic prostate cancer" (Guise and Chirgwin, Co-PIs, 10% effort). Awarded: 02/01/03-3/31/08.
2. National Institutes of Health (NCI), "Breast cancer osteolysis: PTHrP regulation by TGFβ". (R01-CA69158-11; Guise, PI, 20% effort). Awarded: 04/01/01-03/31/06.

CONCLUSIONS

A central tenet in the field of bone metastases is that the bone microenvironment supplies factors, such as TGF-β, stimulating prostate cancer cell signaling and altering their phenotype.

TGF-β signaling in cancer is however complex and can lead to the activation of numerous genes. We have identified many of these genes by microarray analysis and have validated the gene reported here. PMEPA1 was the most highly upregulated

gene. We cloned the PMEPA1 promoter and gene and mapped the TGFβ response element. We are in the process of overexpressing and silencing PMEPA1 in prostate cancer lines to be tested in vivo.

In vivo experiments determined the effects of a TGFβ RI kinase inhibitor, SD-208, on the development and progression of prostate cancer metastases to bone due to PC-3, LuCAP and C42B. Different prostate cancers showed different effects, depending on the radiographic phenotype of the bone metastases. SD-208 improved osteolytic bone metastases due to PC-3, but worsened osteoblastic bone metastases due to LuCAP23.1, with no effect on mixed C42B lesions. The results in C42B were not surprising, since the line is unresponsive to TGFβ. However, the effect of this compound to increase osteoblastic bone metastases is a significant concern. We have initiated an agreement with Eli Lilly to study another TGFβRI kinase that is currently in clinical trials for patients with bone metastases due to all solid tumors.

The p38 MAP kinase inhibitor SD-282 showed no efficacy against any of the bone metastases models and was not studied in additional experiments originally proposed. In particular we concluded that it would be wasteful of experimental animals to carry out combination treatments with this agent, which was the substance of the originally proposed Aim 3.

Overall, we conclude that:

- TGFβ signaling is a useful target for treatment of prostate cancer bone metastases, provided that the tumor cells are responsive to the factor and show components of osteolytic lesions.
- Non-canonical (ie Smad-independent) pathways downstream of the TGFβ receptors, such as p38 MAP kinase, do not appear to be appropriate targets for pharmacological treatment of prostate cancer bone metastases.
- There is no advantage to combined treatment targeting TGFβ receptors and p38 MAP kinase,
- PMEPA1 may be an important target of TGFβ in prostate cancer cells and responsible for potentiating responsiveness of tumor cells in bone to the local actions of bone-released TGFβ.

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APPENDIX

Statement of Work with summaries of progress *in italics*):

Task 1 (Specific Aim 1) – months 01-06. Test TβRI kinase inhibitor at 2 doses (plus untreated controls) against PC3 cells inoculated via intracardiac route (10 mice/group for bone metastasis formation) and subcutaneously (10 mice/group, at higher dose only, for soft tissue growth rate determination). Experiment requires 50 mice. *Completed for bone. Subcutaneous model to be carried out in year 04.*

Task 2 (Specific Aim 1) – months 07-12. Analyze bone and tumor parameters from mice from preceding Task 1. *To be completed in year 04.*

Task 3 (Specific Aim 1) – months 07-12. Test TβRI kinase inhibitor at 2 doses (plus untreated controls) (plus untreated controls) against LuCAP23.1 cells inoculated intratibially (10 mice/group for bone responses) and subcutaneously (10 mice/group, at higher dose, only for soft tissue growth rate determination). Experiment requires 50 mice, plus 5 additional mice for the preparation of the xenograft material = 55 total. *Completed for bone. Subcutaneous model was carried out, but dispersed cells entirely failed to form tumors.*

Task 4 (Specific Aim 1) – months 13-18. Analyze bone and tumor parameters from mice from preceding Task 3. *To be completed in year 04.*

Task 5 (Specific Aim 1) – months 13-18. Test TβRI kinase inhibitor at 2 doses (plus untreated controls) against C4-2B cells inoculated intratibially (10 mice/group for bone responses) and subcutaneously (10 mice/group, at higher dose only, for soft tissue growth rate determination). Experiment requires 50 mice. *Completed for bone. Subcutaneous model eliminated as unnecessary*.

Task 6 (Specific Aim 1) – months 19-24. Analyze bone and tumor parameters from mice from Task 5. *To be completed in year 04.*

Task 7 (Specific Aim 1) – months 01-06. Test p38 MAPK inhibitor at 2 doses (plus untreated controls) against PC3 cells inoculated via intracardiac route (10 mice/group for bone metastasis formation) and subcutaneously (10 mice/group, at high dose only, for soft tissue growth rate determination). Experiment requires 50 mice. *Completed for bone. Subcutaneous model eliminated as unnecessary*.

Task 8 (Specific Aim 1) – months 07-12. Analyze bone and tumor parameters from mice from preceding Task 7. *To be completed in year 04.*

Task 9 (Specific Aim 1) – months 07-12. Test p38 MAPK inhibitor at 2 doses (plus untreated controls) against LuCAP23.1 cells inoculated intratibially (10 mice/group for bone responses) and subcutaneously (10 mice/group, at higher dose only, for soft tissue growth rate determination). Experiment requires 30 mice, plus 5 additional mice for the passage of the xenograft material = 55 total. *Completed for bone. Subcutaneous model eliminated as unnecessary, as well as technically impractical.*

Task 10 (Specific Aim 1) – months 13-18. Analyze bone and tumor parameters from mice from preceding Task 9. *Completed for bone*.

Task 11 (Specific Aim 1) – months 13-18. Test p38 MAPK inhibitor at 2 doses (plus untreated controls) against C4-2B cells inoculated intratibially (10 mice/group for bone responses) and subcutaneously (10 mice/group, at higher dose only, for soft tissue growth

rate determination). Experiment requires 50 mice. *Completed for bone. Subcutaneous model eliminated as unnecessary.*

Task 12 (Specific Aim 1) – months 19-24. Analyze bone and tumor parameters from mice from Task 11. Completed for bone.

Task 13 (Specific Aim 2) – months 01-12. Isolate mRNAs from PC3 and C4-2B cells grown +/- TGFβ and +/- TβRI kinase and p38 MAPK inhibitors at 1 dose each. Analyze RNAs by Affymetrix gene array and process data. *Simplified experiment focusing on PC3 cells treated +/- TGFβ completed, identifying PMEPAS1 as most up-regulated mRNA.*

Task 13a (Specific Aim 2) – months 13-18. Validate genes identified in previous Task 13 by RT-PCR analysis of mRNAs prepared in that Task. *Completed.*

Task 14 (Specific Aim 2) – months 18-24. Generate and characterize stable cell lines of PC3 and C4-2B cells overexpressing FLAG-tagged PMEPA1 protein and, as practical, one or more other candidate factors identified in the previous two Tasks 13 & 13a. *Simplified version of Task completed, focusing on PMEPA1 in PC3 cells.*

Task 15 (Specific Aim 2) – months 25-30. Carry out animal experiments as in Tasks 1 and 5 with control and PMEPA1 overexpressing cell lines (2 cell lines each for PC3 and C4-2B) from previous Task 15. 20 mice per cell line (10 for tumor formation in bone; 10 for subcutaneous growth) = 80 mice total. *Simplified version of Task 15 to be done in year 04, focusing on PMEPA1 in PC3 cells*.

Task 16 (Specific Aim 2) – months 31-36. Analyze bone and tumor parameters from mice from preceding Task 15. *To be done in year 04*.

Task 17 (Specific Aim 3) – months 19-24. Test TβRI and p38 MAP kinase inhibitors singly and combined at optimized doses, plus an untreated control, against PC3 cells inoculated via intracardiac route (10 mice/group for bone metastasis formation). Experiment requires 40 mice. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 18 (Specific Aim 3) – months 25-30. Analyze bone and tumor parameters from mice from preceding Task 17. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 19 (Specific Aim 3) – months 21-26. Test TβRI & p38 MAP kinase inhibitors singly and combined at optimized dose, plus an untreated control, against LuCAP23.1 cells inoculated intratibially (10 mice/group for bone responses. Experiment requires 40 mice, plus 5 additional mice for the passage of the xenograft material = 45 total. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 20 (Specific Aim 3) – months 27-32. Analyze bone and tumor parameters from mice from preceding Task 19. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 21 (Specific Aim 3) – months 25-30. Test TβRI & p38 MAP kinase inhibitors singly and combined at optimized dose, plus an untreated control, against C4-2B cells inoculated intratibially (10 mice/group for bone responses). Experiment requires 40 mice. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 22 (Specific Aim 3) – months 31-36. Analyze bone and tumor parameters from mice from preceding Task 21. *Task abandoned due to lack of efficacy of p38 MAP kinase inhibitor against bone metastases.*

Task 23 (Specific Aims 1-3) – months 03-36. Analyze data, prepare manuscripts and reports. *Continuing with year four no-cost extension.*

Total animal usage: All Aims use male C57/black6 nu/nu mice. Task 1, 50 mice; task 3, 55 mice; task 5, 50 mice; task 7, 50 mice; task 9, 55 mice; task 11, 50 mice; task 15, 80 mice; task 17, 40 mice; task 19, 45 mice; task 21, 40 mice. Total mice over 3 years = 515.